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Structure Determination of Partially Deuterated Carotenoids from Intrinsically Labeled Vegetables by HPLC-MS and ^{1}H NMR

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The structures of biosynthetic deuterated carotenoids in labeled vegetables were investigated: (*all-E*)-lutein and (*all-E*)-β-carotene from spinach, and (*all-E*)-β-carotene and (*all-E*)-α-carotene from carrots. The vegetables were grown hydroponically using a nutrient solution enriched with deuterium oxide (D_2O) and were extracted using matrix solid-phase dispersion (MSPD). Deuterium enrichment in the carotenoid molecules was determined by liquid chromatography−mass spectrometry (LC-MS). (*all-E*)-Lutein and (*all-E*)-β-carotene in spinach showed partial deuteration from $^2H_1$ to $^2H_{12}$, with the abundance maximum at $^2H_5$. (*all-E*)-β-Carotene and (*all-E*)-α-carotene from carrots showed partial deuteration from $^2H_1$ to $^2H_{17}$, with the abundance maximum at $^2H_{11}$. The ^{1}H NMR spectra of the four deuterated carotenoids showed additional signals for all methyl groups and decreased signal intensity for the olefinic protons and the methylene protons in the ring. These differences are due to isotopic effects and are based on the substitution of protons by deuterium atoms. The deuteration was distributed randomly throughout the carotenoid molecules.

KEYWORDS: Carotenoids; deuterated vegetables; HPLC-APCI/MS; ^{1}H NMR

INTRODUCTION

Vegetables grown hydroponically in 15−30 atom-% D_2O (*1*) have been developed for nutritional studies in humans. Labeled spinach, broccoli, collard greens, tomatoes, and carrots have all been grown successfully (*2*). These vegetables are intrinsically labeled with deuterium and have been used to study vitamin K absorption and transport (*3*, *4*), lutein bioavailability (*5*), lycopene absorption and metabolism (*6*), and β-carotene to retinol conversion (*7*). GC-MS (*3*, *4*) and LC-MS (*5*−*7*), and GC-C-IRMS (*8*) have been employed to detect the labeled compounds in human blood serum samples. Kale also has been intrinsically and uniformly labeled with ^{13}C in order to study vitamin K and carotenoid absorption in humans (*9*).

Plants grown in a mixture of deuterated and natural abundance water form partially deuterated compounds during biosynthesis (*1*, *2*). The deuterium enrichment in plants depends on the percent D_2O in water and the metabolic processes in the plant resulting in a set of isotopomers for each hydrogen-containing compound. Although successful human nutritional studies have been carried out, it has not yet been determined where the deuterium atoms are located within the molecules that have been labeled during plant growth. Further, there has been a concern about the potential effects of the nonuniformity of the labeling on the biological action of carotenoids. Previously, Goodwin (*10*) described the biosynthesis of carotenoids from the formation of the basic C_5 terpenoid precursor, isopentenyl pyrophosphate (IPP), to lycopene and finally the conversion into the various naturally occurring carotenes and xanthophylls. Experiments with ^{14}C-labeled acetate in the biosynthesis of (*all-E*)-β-carotene show a distribution of ^{14}C-atoms in the molecule, consistent with the repeating pattern in IPP (*11*). In the intrinsically labeled vegetables of our study, the positions of the deuterium atoms in IPP are unknown and theoretically can be inserted at each hydrogen atom position in the IPP molecule during biosynthesis, such that they will show this pattern later in the carotenoid molecule.

The investigation of many biological tissues, such as the analysis of carotenoids in vegetables, needs to be performed using sophisticated analytical methods. Carotenoids are extremely sensitive to light and air due to their highly conjugated system of double bonds (**Figure 1**). For that reason, an optimized combination of analytical extraction, separation, and detection techniques has to be used. The extraction of carotenoids from the plant material has been performed by liquid−liquid extraction (LLE) (*34*, *35*), solid-phase extraction SPE (*36*−*38*) and matrix solid-phase dispersion, MSPD (*12*−*14*). MSPD is based on SPE and has been designed for the extraction of analytes from solid and viscous samples. In comparison to

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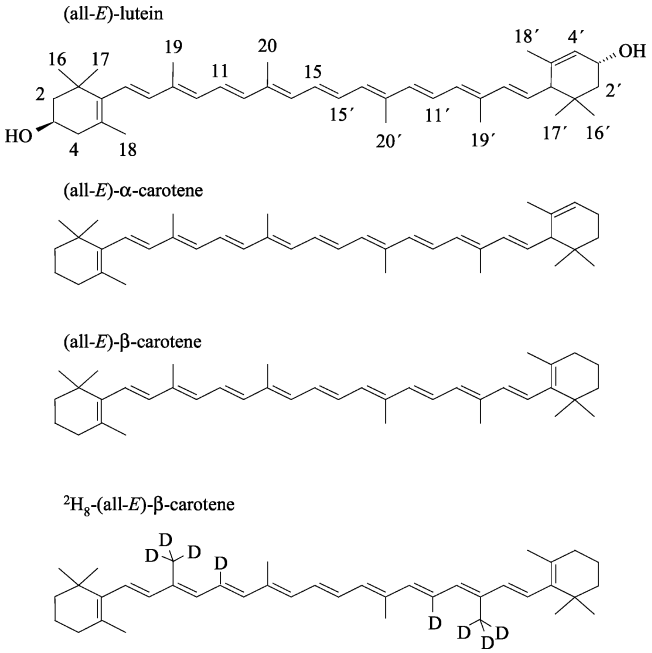

Figure 1. Chemical structures of (*all-E*)-lutein, (*all-E*)-α-carotene, (*all-E*)-*β*-carotene, and $^2H_8$-(*all-E*)-*β*-carotene.

LLE and SPE, the amount of solvent and also the required time for the extraction are decreased, and higher concentrations of the carotenoids are extracted. High concentrations of carotenoids are necessary for successful NMR measurements.

For the HPLC separation of naturally occurring carotenoids and carotenoid isomers, C_{30}-bonded silica phases have been employed extensively and their use has been reviewed (*33*). These C_{30} phases show much higher shape selectivity for shape-constrained natural compounds as carotenoid stereoisomers and enhanced sample-loading capacity compared to C_{18} materials. Since the introduction of C_{30} phases by Sander, the synthesis of C_{30} phases has been further improved and detailed NMR studies have been carried out (*15*, *16*).

The technique of HPLC-APCI/MS with its high sensitivity is usually suitable for a fast and unambiguous structural elucidation of carotenoids. Within one chromatographic run, all peaks can be identified and assigned to the different carotenoids. APCI/MS allows the ionization of nonpolar carotenoids in positive as well as in negative ion mode with little fragmentation (*18*−*22*). While HPLC-APCI/MS allows the determination of different carotenoids and the detection of deuterium enrichment in the labeled carotenoids, it is not possible to distinguish between stereoisomers or the position of labeling in the molecule. Due to the energy input during the ionization and fragmentation, the deuterium atoms will be scrambled along the hydrocarbon chain of (*all-E*)-*β*-carotene (*23*).

Therefore, to obtain a full and unambiguous structure elucidation, NMR spectroscopy is absolutely necessary. In particular, NMR spectroscopy has the capability to distinguish between structural and conformational isomers and can detect the position of the deuterium atoms in the carotenoid molecule. Since NMR is not known for its sensitivity, the development of solenoidal-type microcoil NMR probes is able to overcome this limitation (*24*). The solenoidal-type is constructed by directly wrapping the RF coil around a capillary column and placing it into a container with susceptibility-matching fluid transverse to the magnetic field (*25*). Therefore, the solenoidal probe design shows severalfold better sensitivity than saddle-shaped coils and has successfully been utilized for on-line coupled capillary HPLC NMR measurements (*26*). Due to the small active volume (1.5 μL) of the microcoil NMR probe, mass-limited samples can be dissolved in smaller solvent volumes, thus enhancing their concentration.

This study was undertaken to determine the deuterium distribution of partially deuterated carotenoids in intrinsically labeled vegetables, using MSPD extraction, HPLC-APCI/MS and ^{1}H NMR.

MATERIALS AND METHODS

Chemicals. For HPLC separations, acetone (LiChrosolv gradient grade) from Merck (Darmstadt, Germany) and deionized water from a Milli-Q water purification system (Millipore AS, Bedford, MA) were used. NMR measurements were carried out in chloroform-$^2H_1$ (Uvasol) from Merck (Darmstadt, Germany). (*all-E*)-lutein and $^2H_8$-(*all-E*)-*β*-carotene were a gift of BASF AG (Ludwigshafen, Germany) and (*all-E*)-*β*-carotene was purchased from Fluka (Taufkirchen, Germany). For peak assignment in ^{1}H NMR spectra, 100 μg of the pure substances were dissolved in 100 μL of chloroform-$^2H_1$. The extraction of the undeuterated and deuterated carotenoids from plant material was performed utilizing MSPD with a silica-based octadecyl sorbent material (C_{18} end capped) from IST Ltd (Hengoed Mid Glamorgan, UK).

Sample Preparation. The intrinsically labeled spinach (cultivar Melody) and carrots (cultivar Lucky BF Hybrid) were grown hydroponically at the USDA/ARS Children's Nutrition Research Center in Houston, TX, employing a nutrient solution enriched with 15 atom-% D_2O for spinach and 30 atom-% D_2O for carrots (*1*). For both crops, seeds were germinated in deionized water (H_2O), and 4-d-old seedlings were placed in their respective D_2O-enriched nutrient solution. Plants were maintained on D_2O solutions until harvest; growth periods were 4 weeks for spinach and 10 weeks for carrots. After harvest, spinach and carrots were chopped into small pieces, deep frozen with liquid nitrogen and stored at −80 °C. One half gram of the samples was ground with 1.5 g of C_{18} (end capped) MSPD-material to a dry homogeneous powder. The mixture was loaded into an empty SPE column and pressed between two frits. The column was washed with 10 mL of deionized water and the carotenoids were extracted with 4 mL of acetone. After evaporation of acetone under a nitrogen stream, the extract was redissolved in 200 μL of acetone, guaranteeing a high concentration of the carotenoids.

Chromatography. Analyses were carried out on an HP1100 HPLC system (Agilent Technologies, Waldbronn, Germany) using a UV detector monitoring at 450 nm. The separations were performed on a 250 × 4.6 mm ProntoSil C_{30} stainless steel column (Bischoff, Leonberg, Germany). The particle size was 3 μm and the average pore diameter 200 Å. The separations of the carotenoids from the MSPD extracts were performed using a mobile-phase gradient elution program at a flow rate of 1 mL min^{-1}. Initially, an isocratic mixture of acetone/water (86:14, v/v) was used for 21 min, followed by a 4-min linear gradient to acetone/water (97:3, v/v), maintaining this solvent composition until the end of the separation at 40 min. A 50 μL volume of the MSPD extracts was injected four times. The undeuterated and deuterated (*all-E*)-lutein, (*all-E*)-α-carotene, and (*all-E*)-*β*-carotene were collected. After fraction collection the solvent was evaporated, and the carotenoids were redissolved in 50 μL of chloroform-$^2H_1$.

HPLC-MS. Mass spectrometry was performed on a Bruker Esquire 3000plus LC-MS$^{(n)}$-System (Bruker Daltonik, Bremen, Germany) equipped with an APCI interface and an ion trap. The HPLC-APCI/MS coupling was accomplished using an HP1100 system (Agilent Technologies, Waldbronn, Germany) injecting 10 μL.

The mass spectra were recorded in the mass region of *m*/*z* 400−700. The detection was performed using APCI in the positive ion mode. The voltage of the corona needle was set to 4 kV. Nitrogen was used as drying as well as carrier gas at a flow rate of 5 L/min with a nebulizer pressure of 65 psi. The ionization chamber and dry gas temperature were held at 300 °C. The compound stability and trap drive level were set to 75%. The chromatographic conditions were the same as in the analytical separation described above.

^{1}H NMR. All NMR experiments were recorded using a Bruker AMX 600 spectrometer (Bruker, Rheinstetten, Germany). ^{1}H NMR spectra

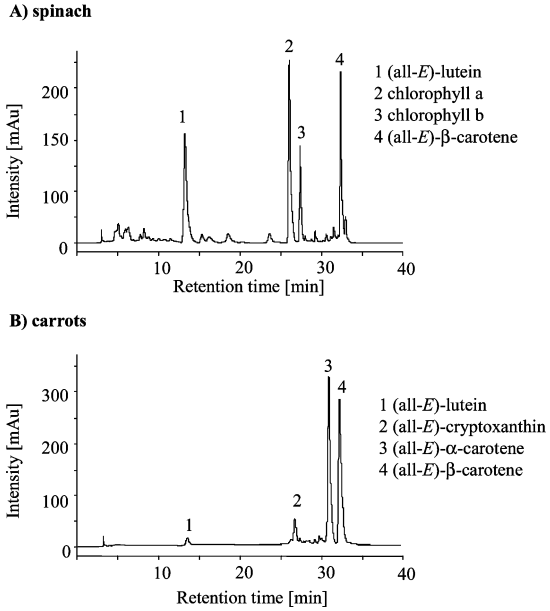

Figure 2. HPLC–UV chromatograms of the carotenoids and chlorophylls separated from spinach leaves (A) and carrot roots (B).

of biosynthesized carotenoids were recorded utilizing syringe flow-injection to a $^{1}H-^{13}C$ inverse microcoil NMR probe (Protasis/MRM, Savoy, IL) with an active volume of 1.5 μL. ^{1}H NMR spectra of synthetic $^{2}H_{8}$-(*all-E*)-β-carotene and standard were recorded in a 5 mm tube NMR probe (Bruker, Rheinstetten, Germany). All experiments were recorded with the pulse program zg30 without solvent suppression. For the ^{1}H NMR spectra, 1 k transients were accumulated (4 s per scan; AQ 3 s, D1 1 s) with a time domain of 32 k and a sweep width of 5376 Hz. The temperature was set to 298 K. All NMR data were processed with 1D WINNMR (Bruker, Rheinstetten, Germany). Prior to Fourier transformation, the FID was multiplied by an exponential function with a line-broadening factor of 0.5 Hz.

RESULTS AND DISCUSSION

The extraction of the carotenoids from the plant material (spinach, carrots) was performed using MSPD, and the carotenoids were separated on a C_{30} column as shown in **Figure 2**. The upper chromatogram shows the chromatographic separation of the spinach MSPD extract, the lower chromatogram the separation of the carrot MSPD extract. Spinach contains mainly (*all-E*)-lutein, (*all-E*)-β-carotene, chlorophyll a and b, and also some minor compounds, which represent the various Z stereoisomers (*14*). In contrast, carrot contains mainly (*all-E*)-α-carotene, (*all-E*)-β-carotene, and only a small amount of (*all-E*)-lutein (*27*).

HPLC-APCI/MS was performed to determine the extent of deuteration of the different carotenoids. In many previous experiments (*5*, *12*, *18*−*22*) on carotenoids, APCI/MS has produced abundant protonated molecules $[M + H]^+$ in the mass spectrum. **Figure 3** shows the protonated molecule sections of the APCI mass spectra of the four carotenoids. All the mass spectra show an approximately Gaussian distribution of carotenoid isotopomers biosynthesized by the plants. This is consistent with previous experiments (*3*,*5*). The deuterated (*all-E*)-lutein and (*all-E*)-β-carotene from labeled spinach (15 atom-% D_2O) show a mass/charge ratio range indicating isotopomers from $^{2}H_1$ to $^{2}H_{12}$. The most abundant one is $^{2}H_5$ (**Figure 3A**).

The undeuterated (*all-E*)-lutein from spinach shows a peak at *m*/*z* 551 $[M + H - H_2O]^+$ (data not shown). The deuterated (*all-E*)-lutein from spinach has an isotopomer distribution from *m*/*z* 552 to 562 with the most abundant at *m*/*z* 556 $[M + (^{2}H_5) + H - H_2O]^+$. The undeuterated (*all-E*)-β-carotene from spinach shows a peak at *m*/*z* 537 $[M + H]^+$ (data not shown), and the deuterated (*all-E*)-β-carotene from spinach has an isotopomer distribution from *m*/*z* 538 to 549 with the most abundant at *m*/*z* 542 $[M + (^{2}H_5) + H]^+$. In comparison, the deuterated (*all-E*)-α-carotene and (*all-E*)-β-carotene from carrots (30 atom-% D_2O) show a range of isotopomers from $^{2}H_5$ to $^{2}H_{17}$ with the most abundant one at $^{2}H_{11}$ (**Figure 3B**).

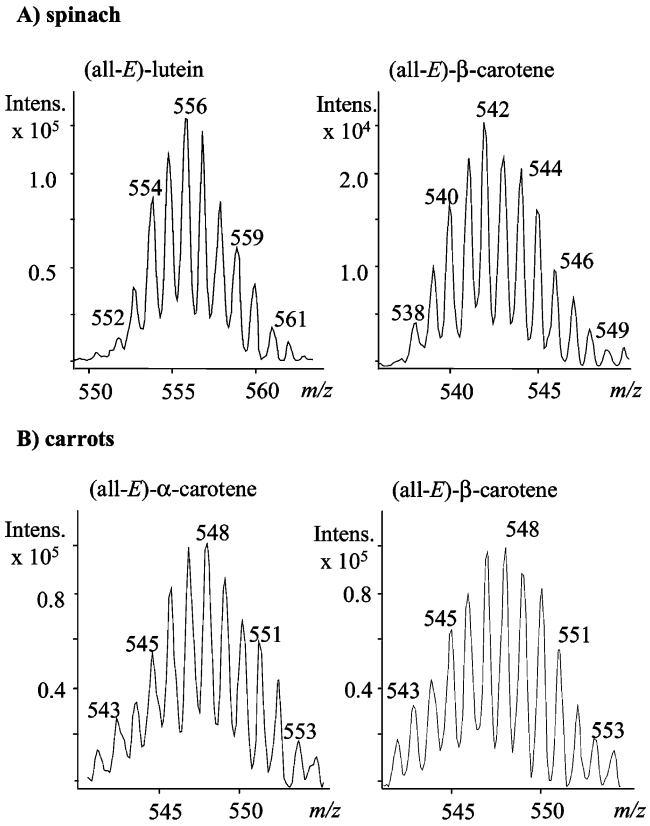

Figure 3. The Gaussian isotopomer distribution of the deuterated (*all-E*)-lutein and (*all-E*)-β-carotene from labeled spinach (A) and (*all-E*)-α-carotene and (*all-E*)-β-carotene from labeled carrots (B).

The undeuterated (*all-E*)-α-carotene from carrots shows a peak on *m*/*z* 537 $[M + H]^+$ (data not shown). The deuterated (*all-E*)-α-carotene from carrots has an isotopomer distribution from *m*/*z* 542 to 554 with the most abundant at *m*/*z* 548 $[M + (^{2}H_{11}) + H]^+$. The undeuterated (*all-E*)-β-carotene from carrots shows a peak at *m*/*z* 537 $[M + H]^+$ (data not shown), and the deuterated (*all-E*)-β-carotene from carrots has an isotopomer distribution from *m*/*z* 542 to 554 with the most abundant at *m*/*z* 548 $[M + (^{2}H_{11}) + H]^+$. The extent of deuteration in the carrots is greater than in spinach due to the fact that it was grown in more highly enriched deuterated water (*1*, *2*).

NMR spectroscopy has the capability to detect the exact position of deuteration within the carotenoid molecule, so ^{1}H NMR spectra were collected. There are two possible arrangements of deuterium atoms in the intrinsically labeled carotenoids, a random distribution of deuterium atoms within the whole carotenoid molecule, or a nonrandom distribution focused more heavily in specific parts of the carotenoid molecule, e.g. in the olefinic section or in the methyl groups. To compare the NMR spectra of carotenoids from undeuterated and deuterated samples, one needs to ensure the same concentrations of carotenoids in the both samples, and also must employ the same NMR recording parameters, e.g. solvent composition, transients, time domain, and sweep width, for both samples.

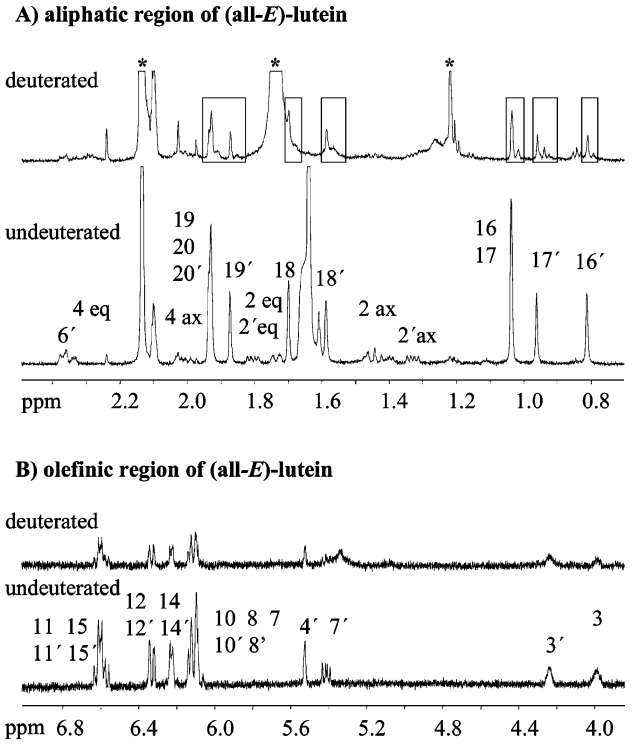

Figure 4. ^{1}H NMR spectra (600 MHz, 1.5 μL solenoidal microprobe) of (*all-E*)-lutein from labeled spinach and unlabeled standard in chloroform-$^2H_1$. (A) Aliphatic region of (*all-E*)-lutein, with the lower spectrum depicting undeuterated standard and the upper spectrum depicting deuterated (*all-E*)-lutein from spinach extract. (B) Olefinic region of (*all-E*)-lutein, with the lower spectrum depicting undeuterated standard and the upper spectrum depicting deuterated (*all-E*)-lutein from spinach extract (*interfering signals).

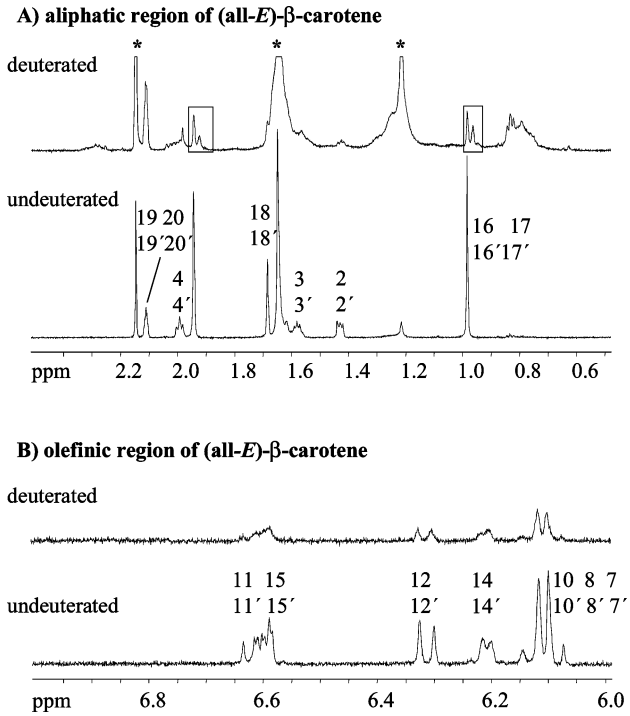

Figure 5. ^{1}H NMR spectra (600 MHz, 1.5 μL solenoidal microprobe) of (*all-E*)-β-carotene from labeled carrots and unlabeled standard in chloroform-$^2H_1$. (A) Aliphatic region of (*all-E*)-β-carotene, with the lower spectrum depicting undeuterated standard and the upper spectrum depicting deuterated (*all-E*)-β-carotene from carrot extract. (B) Olefinic region of (*all-E*)-β-carotene, with the lower spectrum depicting undeuterated standard and the upper spectrum depicting deuterated (*all-E*)-β-carotene from carrot extract (*interfering signals).

NMR requires a much greater concentration of carotenoids than mass spectrometry. To increase the concentration of the carotenoids and guarantee the same recording conditions, the fractions of (*all-E*)-lutein, (*all-E*)-α-carotene, and (*all-E*)-β-carotene were collected after separation on a C_{30} column. After evaporating the solvent under a nitrogen stream and redissolving in chloroform-$^2H_1$, the standards and the four collected deuterated carotenoids were injected with a into the microcoil NMR probe and all spectra were recorded under equivalent experimental conditions. The spectra of (*all-E*)-lutein from spinach and (all E)-β-carotene from carrots are depicted in **Figures 4** and **5**. Interfering signals (probably from the solvent) in the NMR spectra are noted with an asterisk in **Figures 4**, **5**, and **6**.

(*all-E*)-Lutein, (*all-E*)-β-carotene, and (*all-E*)-α-carotene are well-studied carotenoids and their ^{1}H NMR spectra are well described (*28*−*31*). **Figure 4A** shows the aliphatic part of the undeuterated (lower) and deuterated (upper) (*all-E*)-lutein from spinach. Because (*all-E*)-lutein is an unsymmetrical molecule, there are several signals for methyl groups 16′ to 19′ and also for the methylene groups in the ring. In the spectra of undeuterated (*all-E*)-lutein, the methyl group 16′ has a singlet at 0.82 ppm, 17′ at 0.96 ppm, 18′ at 1.59 ppm, 19′ at 1.87 ppm, and 18 at 1.70 ppm. The integration values for all three are consistent with the number of protons in these methyl groups. The methyl groups 16 and 17 have a singlet at 1.04 ppm with an integration value of six and the methyl groups 19 and 20/20′ a singlet at 1.93 ppm with an integration value of nine. In the spectrum of deuterated (*all-E*)-lutein there arise additional signals at a lower chemical shift next to these methyl group signals (gray boxes). They show smaller intensities and in some cases more or less fine structure. These additional signals arise from the partly deuterated methyl groups in the carotenoid molecule and the fine structure from proton/deuterium atom coupling in these partly deuterated methyl groups. Due to the substitution of protons by deuterium atoms within the methyl groups, the chemical and magnetic environment changes; this results in a slightly different local magnetic field for the residual methyl protons (*32*). The substitution of a lighter proton by a heavier deuterium atom leads to more shielding and this isotopic effect leads to additional NMR signals at a lower frequency, with decreased signal intensities and integration values. The integration values of the spectrum of deuterated (*all-E*)-lutein cannot be used for a comparison with the standard spectrum, because the integration values are a sum of all the isotopomers in the deuterated (*all-E*)-lutein sample.

The mass spectra in **Figure 3** show a mixture of different carotenoid isotopomers with an approximately Gaussian distribution. In the sample with undeuterated (*all-E*)-lutein all the molecules have the same number of protons in undeuterated methyl groups, but in the sample with partly deuterated (*all-E*)-lutein from spinach we can find molecules with different deuterated methyl groups, e.g. CH_3, CDH_2, CD_2H, or CD_3. The integration value can only be used for a comparison between methyl groups in this sample. They show the same proportions between the methyl groups as in the undeuterated sample, which means that all the methyl groups show similar deuterium enrichment and no group is more favored than another group.

In **Figure 4A** the signals of the methylene groups 2/2′ and 4, and proton 6′ show a multiplet structure in the standard spectrum and also in the spectrum of the deuterated (*all-E*)-lutein. The only difference in this section of the spectra is that

the partly deuterated signals are less intense. The proportions between the proton signals are equal in both spectra. The multiplets in the spectrum of deuterated (*all-E*)-lutein are very complex for these protons, because they show couplings between protons and deuterium atoms in the neighboring groups and couplings to a proton or deuterium atom in the same group. The decreased signal intensities come from the partial substitution of protons by deuterium atoms at these positions.

In **Figure 4B** the olefinic region of the spectra from (*all-E*)-lutein is depicted. In detail, we found in the olefinic region of the standard spectrum a singlet at 5.53 ppm for proton 4′, two doublets at 6.34 ppm for protons 12/12′ and at 6.23 ppm for protons 14/14′, a doublet of doublets at 5.42 ppm for proton 7′ and two multiplets at 6.61 ppm for protons 11/11′ and 15/15′ and at 6.12 ppm for protons 7, 8/8′ and 10/10′. There are also two broad signals at 4.23 ppm for proton 3′ and at 3.99 ppm for proton 3, with the hydroxyl group on the same carbon atom. The integration values of the olefinic protons are one for 4′ and 7′, two for 12/12′ and 14/14′, four for 11/11′ and 15/15′, and five for 7, 8/8′ and 10/10′. Each signal of 3 and 3′ has an integration value of one. Similar to the methylene groups in the aliphatic part there is a decrease in signal intensities for the olefinic protons in the deuterated (*all-E*)-lutein relative to the undeuterated lutein. The proportions of the integration values among the different signals in the spectrum of deuterated (*all-E*)-lutein are identical to those in the spectrum of undeuterated lutein. These results show that there are no preferred positions in the lutein molecule for a substitution of a proton by a deuterium atom.

The spectra of (*all-E*)-*β*-carotene (**Figure 5**) from carrots show fewer signals due to the symmetric molecular structure, but the deuteration behavior is identical to (*all-E*)-lutein. The standard spectrum methyl groups 19/19′ and 20/20′ have a singlet at 1.95 ppm with an integration value of twelve, methyl groups 18/18′ a singlet at 1.70 ppm with an integration value of six, and methyl groups 16/16′ and 17/17′ a singlet at 1.00 ppm with an integration value of twelve. The spectrum of the deuterated (*all-E*)-*β*-carotene also has additional signals at a lower chemical shift next to the methyl groups 16/16′, 17/17′ and 19/19′, 20/20′ (gray boxes). These additional signals show also the presence of partially deuterated methyl groups as in the (*all-E*)-lutein spectrum. The higher intensities of the additional signals for methyl groups 19/19′and 20/20′ in comparison to the (*all-E*)-lutein spectrum is due to higher deuterium enrichment, $^2H_1$ to $^2H_{12}$ for spinach and $^2H_5$ to $^2H_{17}$ for carrots. The signal for methyl groups 18/18′ is overlapped with an interfering signal (probably from the solvent) and cannot be unambiguously evaluated. The methylene groups 2/2′, 3/3′, and 4/4′ in the spectrum of deuterated (*all-E*)-*β*-carotene show decreased signal intensities, and the broadened multiplets in comparison to the spectrum of undeuterated *β*-carotene. These effects on the signals are due to the partial deuteration of the molecule and show the influence of an isotopic effect (*32*). The decreased signal intensity derives from the lower number of protons in this group. The broadening effect is caused by the change of the chemical and magnetic environment of the group due to the partial exchange of several neighbored protons by deuterium atoms. Due to the different couplings between protons and deuterium atoms, additional slightly shifted signals arise that caused the signal broadening. Also the olefinic protons have decreased signal intensities in comparison to the to the spectrum of undeuterated *β*-carotene. Finally, the proportions of integration values among methyl groups, methylene groups, and olefinic protons show that there are no preferred positions in the *β*-carotene molecule for substitution of a proton by a deuterium atom. The spectra of (*all-E*)-*β*-carotene from spinach and (*all-E*)-α-carotene from carrots are not depicted here, but they show exactly the same changes due to deuterium enrichment as (*all-E*)-lutein from spinach and (*all-E*)-*β*-carotene from carrots.

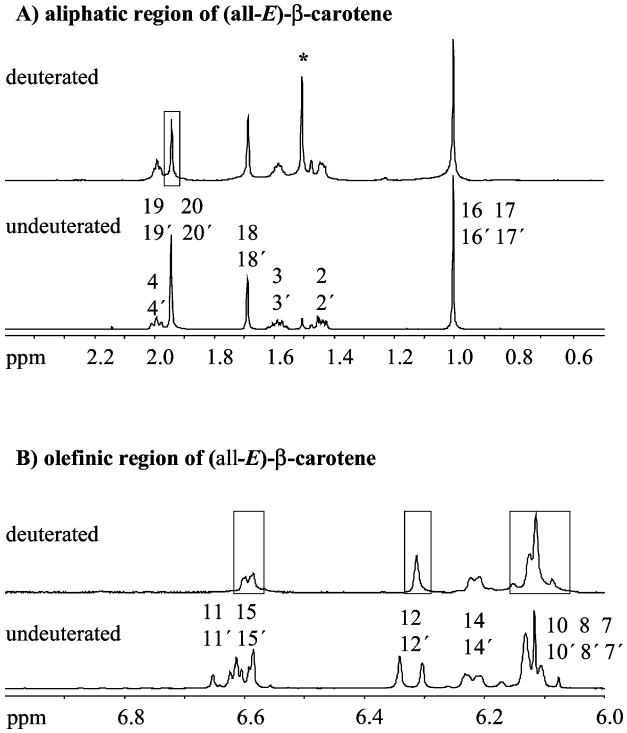

Figure 6. ^{1}H NMR spectra (600 MHz, 5 mm tube) of synthetic labeled $^2H_8$-(*all-E*)-*β*-carotene and unlabeled standard in chloroform-$^2H_1$. (A) Aliphatic region of (*all-E*)-*β*-carotene, with the lower spectrum depicting undeuterated and the upper spectrum depicting deuterated (*all-E*)-*β*-carotene. (B) Olefinic region of (*all-E*)-*β*-carotene, with the lower spectrum depicting undeuterated and the upper spectrum depicting deuterated (*all-E*)-*β*-carotene (* interfering signals).

The ^{1}H NMR spectrum of a synthetic labeled carotenoid look completely different from the ^{1}H NMR spectra of biosynthesized labeled carotenoids. **Figure 6** shows a ^{1}H NMR spectrum of synthetic $^2H_8$-(*all-E*)-*β*-carotene. The molecule has two deuterium atoms in the olefinic part in position 11 and 11′ and six deuterium atoms in the aliphatic part in methyl groups 19 and 19′. The aliphatic region shows no additional signals as in biosynthesized labeled carotenoids. The only difference from the standard spectrum is the integration value of the signal for methyl groups 19/19′ and 20/20′, because the fully deuterated methyl groups show no signals in the spectrum. The integration value changed from twelve for four methyl groups to six for the two visible methyl groups 20 and 20′. Here only one type of molecule is being measured and not a mixture of different isotopomers so the integration values can be used. All other methyl and methylene signals in the aliphatic part are identical in appearance, and the integration values are the same.

The olefinic part of the spectrum shows some more differences in comparison to the standard spectrum. The olefinic protons 11 and 11′ show couplings with protons 10 and 12, and 10′ and 12′ in the standard spectrum. Due to the deuteration of this position, there are no couplings between the other protons. Couplings between the protons 10/10′ and 12/12′ with deuterium atoms 11/11′ are possible, but the coupling constants are significantly smaller than the proton/proton couplings (*J*

(H,H) ≈ 6.5J (H,D)). This means, in some cases that these couplings are not resolved, but they exist.

In the standard spectrum, protons 12 and 12′ show a doublet at 6.33 ppm due to the coupling with proton 11 and 11′ and an integration value of two for these two protons. In the spectrum of the labeled carotenoid, only a singlet is visible with an integration value of two. This means that the number of protons is the same: two for protons 12 and 12′. There are no couplings between protons 11 and 11′ because they are both labeled with deuterium atoms. The same behavior shows protons 10 and 10′ in the multiplet at 6.14 ppm. The singlet is not clearly visible in the spectrum of the labeled carotenoid, but the left part of the multiplet fine structure has changed and the integration value is six in both spectra. The multiplet for protons 11/11′ and 15/15′ at 6.62 ppm has changed now to a doublet for protons 15 and 15′. Also the integration value has changed from four to two for protons 15 and 15′. The signal and the integration values for protons 14 and 14′ at 6.22 ppm are identical in both spectra.

Our results with random distributions of the deuterium atoms in the biosynthesized labeled carotenoid molecule is in contrast to the uniquely labeled positions observed by Goodwin (*10*) and Lotspeich et al. (*11*) for (*all-E*)-β-carotene derived from ^{14}C-labeled acetate. In the present study, the hydroponic culture of plants with heavy water allows deuterium to enter the primary metabolism prior to the synthesis of acetate (and before IPP), and thus deuterium subsequently appears in all possible positions within the biosynthesized carotenoids.

The combined use of MSPD, a powerful but also mild and rapid extraction technique, with an HPLC separation method based on a C_{30} stationary phase enabled the analysis and structure elucidation of biosynthesized deuterated carotenoids from plant material. The determination of the isotopomer distribution of the most abundant carotenoid molecules was accomplished by employing HPLC-APCI/MS and the distribution of the deuterium atoms was determined with ^{1}H NMR in a microcoil NMR probe. It was verified that the deuterium atoms show a random distribution over the whole carotenoid molecule independent from carotenoid type, isotopomer distribution, and plant material. Possible differences in the labeling pattern between the symmetric (*all-E*)-β-carotene and the asymmetric (*all-E*)-lutein and (*all-E*)-α-carotene could be not detected. Also the differences in the spectra between synthetically labeled and biosynthesized labeled carotenoids are clearly shown. Utilization of intrinsically labeled vegetables (partial replacement of protons with deuteriums in a random distribution in carotenoids) enables an easy differentiation between isotopomers from the vegetables and endogenous unlabeled carotenoids.

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